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Salmonella Strain Secreting Active Listeriolysin Changes Its Intracellular Localization

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We describe the construction of an attenuated *Salmonella dublin aroA* strain which secretes via the *Escherichia coli* hemolysin secretion machinery an active hybrid cytolysin consisting of listeriolysin from *Listeria monocytogenes* and the C-terminal secretion signal of *E. coli* hemolysin. This hemolytic *S. dublin* strain is partially released into the cytoplasm of the host cell following uptake by J774 macrophage cells, whereas the nonhemolytic control *S. dublin aroA* strain remains in the phagosome.

Facultative intracellular bacteria, like *Salmonella dublin* and *Listeria monocytogenes* studied here, resist intracellular killing by macrophages (13). The modes of intracellular survival of these two bacterial pathogens are different. *S. dublin* remains in the phagosomal vacuole and replicates within this compartment (2a), whereas *L. monocytogenes* quickly enters the cytoplasm of the host cell after lysis of the phagosomal membrane by listeriolysin (4, 14, 17). Replication of the listeriae takes place within the cytoplasm and is accompanied by the polymerization of cellular actin, which promotes active intra- and intercellular movements (17).

Construction of a *Salmonella* strain which secretes active listeriolysin was achieved by cloning the *hly* gene of *L. monocytogenes* EGD (encoding listeriolysin [11]) into the *Escherichia coli* hemolysin determinant such that *hly* was fused in frame to the last 183 bp of the *E. coli* hemolysin gene (*hlyA*). This 3′ part of *hlyA* encodes the C-terminal 61-amino-acid secretion signal of *E. coli* hemolysin ($HlyA_s$) (6–8), which is recognized by the HlyB-HlyD-TolC secretion machinery (10, 19). The 5′ portion of the *hly* gene which encodes the N-terminal signal sequence of listeriolysin was removed in order to avoid interference with the *sec* transport machinery (15). As shown previously, an N-terminal signal sequence considerably inhibits the secretion efficiency of the hemolysin secretion apparatus (6). Primers M432, 5′-*Nsi*I-GCAAAGGATGCATCT GCATTC-3′ (positions 199 to 220), and M431, 5′-*Nsi*I-ACTT TTATGCATATTCGATTGGATTATC-3′ (positions 1711 to 1729), were derived from the published sequence of *hly* (11) and were used to amplify by PCR (16) a 1.5-kb DNA fragment of the *hly* gene. The underlined sequence indicates the *Nsi*I recognition site. PCR was carried out in a Thermal Cycler 60 (Bio-Med, Theres, Germany), for 30 cycles at 94°C for 1 min, 55°C for 45 s, and 72°C for 1 min. The 1.5-kb amplicon, carrying the *hly* gene without the N-terminal signal sequence, was digested with *Nsi*I, gel purified with the Geneclean Kit (Bio 101, La Jolla, Calif.), and cloned into the *Nsi*I site of plasmid pMOhly1 (12, 18). The resulting plasmid pILH1 carried the *hly-hlyA*$_s$ fused gene and the functional *hlyB* and *hlyD* genes required for its secretion (Fig. 1).

Aliquots (0.05 ml) of *L. monocytogenes* EGD, *S. dublin aroA*/pILH1 (encoding the listeriolysin $HlyA_s$ fusion protein [LLO-$HlyA_s$]) and *S. dublin aroA*/pMOhly1 (control carrying the vector plasmid alone) grown in 2× yeast tryptone medium to an optical density of 1.0 were incubated with 0.5% washed sheep erythrocytes in phosphate-buffered saline (PBS) buffer (pH 5.5) at 37°C for 1 h to test the hemolytic activity. The hemolytic activity of the supernatant and that of the whole-cell suspension of the *S. dublin aroA*/pILH1 strain are similar to those of the parental strain *L. monocytogenes* EGD (Table 1). Dextran 8 (30 mM) in PBS (pH 5.5) provides protection against listeriolysin- or hybrid listeriolysin protein-induced lysis in a 30-min hemolysis assay at 37°C. The hemolysis assay was set up with 500 μl of a 2% sheep erythrocyte suspension (PBS [pH 5.5], 60 mM Dextran 8), and 500 μl of culture supernatant (concentration of toxin, 1 to 2 μg/ml) was added to the erythrocytes in a total volume of 1 ml. These preliminary osmotic protection experiments indicate that the two toxins may form pores of similar size in erythrocyte membranes (5). The amount of the hybrid listeriolysin protein secreted into the supernatant by *S. dublin aroA* or *E. coli* K-12 is, however, at least sixfold higher than the amount of listeriolysin secreted into the supernatant by *L. monocytogenes* EGD under the same conditions (Fig. 2), indicating a reduction in the specific activity of this hybrid listeriolysin protein compared with that of the original listeriolysin.

We were interested in the impact of the hybrid listeriolysin protein on intracellular replication of the *S. dublin* strain in phagocytic host cells. Normally, *S. dublin* survives and replicates in the phagosomal vacuole (2a), whereas *L. monocytogenes* evades into the cytoplasm (17). It has been demonstrated that listerolysin is an essential factor for this evasion step, and even *Bacillus subtilis* synthesizing listeriolysin is partially released into the cytoplasm after its uptake by macrophages (1). The question of whether the synthesis of this cytolysin converts the normally vacuole-bound intracellular lifestyle of *S. dublin* into a cytoplasmic one therefore arises. To this end, the *S. dublin aroA* strain was incubated with J774 macrophage cells. The *Salmonella* strain producing the hybrid listeriolysin protein does not cause substantial killing of the J774 macrophages and is taken up efficiently by these host cells. The cytotoxicity was monitored by testing the activity of lactate dehydrogenase present in the medium of J774 cells 3 h after infection with hemolytic *S. dublin* as described by Zychlinsky et al. (20). By electron microscopy of thin sections of infected J774 cells, it was found that at least 15% (39 of 251) of the intracellular

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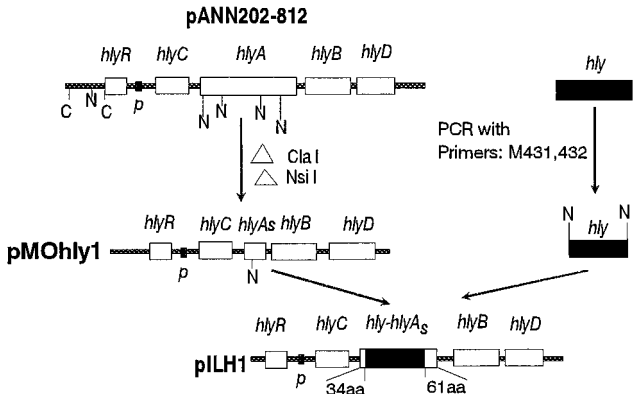

FIG. 1. Construction of recombinant plasmid pILH1. The *hly* gene of *L. monocytogenes* EGD is represented by a black box, and the genes and sequences (*hlyR*) necessary for hemolysin expression in *E. coli* and *S. dublin* are represented by open boxes. Restriction sites are as follows: C, *Cla*I; N, *Nsi*I. The gene sizes are drawn out of scale.

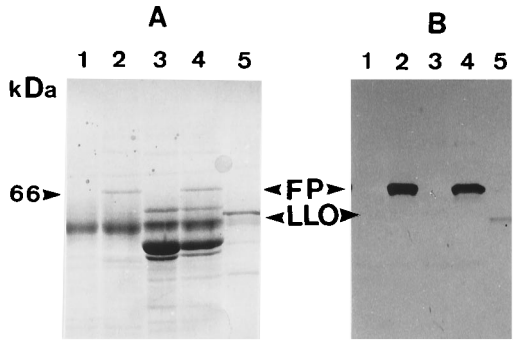

FIG. 2. Identification of the LLO-HlyA$_s$ fusion protein by sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis. Cultures of *E. coli* K-12 and *S. dublin aroA* carrying plasmid pMOhly1 (lanes 1 and 3) or plasmid pILH1 encoding the LLO-HlyA$_s$ protein (lanes 2 and 4) and *L. monocytogenes* (lanes 5) were grown in 2× yeast tryptone medium to a cell density of 7×10^8 cells per ml. (A) Coomassie brilliant blue R250-stained acrylamide gel. Note that the LLO protein (lane 5) is located under the most prominent band (p60 protein) on the gel. (B) Immunoblot of the proteins developed with polyclonal anti-listeriolysin antibodies. In all lanes the supernatant proteins obtained from 1 ml of bacterial culture were loaded. The samples were prepared and analyzed as previously described (6, 8).

microorganisms were localized in the cytoplasm of the macrophages after 3 h of incubation (Fig. 3A). The control strain *S. dublin aroA*/pMOhly1 remained exclusively in the phagosome (all 191 bacteria examined are located in this compartment; Fig. 3B). A recent study (17) on the intracellular survival of *L. monocytogenes* in J774 cells showed that 69% of these bacteria were free in the cytoplasm within 1.30 h after infection. This suggests that the hybrid listeriolysin protein, when secreted by *S. dublin*, can disrupt the phagosomal membrane, but escape into the cytoplasm may not be as efficient as with the listeriolysin of *L. monocytogenes*. One should, however, keep in mind that escape into the cytoplasm by *L. monocytogenes* is caused by listeriolysin and the phosphatidylinositol-specific phospholipase C (*plcA*), which is probably not present in *S. dublin.*

Note that an attenuated *aroA* mutant of *S. dublin* was used in these experiments. For biosafety reasons we could not carry out the same experiments with a wild-type strain of *S. dublin*. The question of whether the isogenic wild-type *S. dublin* strain carrying the hybrid listeriolysin gene may escape and replicate more efficiently in the cytoplasm of infected macrophages therefore cannot be answered. The rate and efficiency of intracellular bacterial growth in the hemolytic strain were not altered compared with that in the nonhemolytic wild-type *aroA* strain (Fig. 4). In both cases, only limited replication of the salmonellae was observed. This may indicate that the cytoplasm does not supply the growth supplements required by the *aroA* mutant. This is not surprising, since the mammalian cells may provide tryptophan, phenylalanine, and tyrosine but not *p*-aminobenzoic acid and 2,3-dihydroxybenzoic acid, metabolites which are essential for bacterial growth but not synthesized in the *aroA* mutant (2). However, even an *E. coli* K-12 wild-type strain (secreting the hybrid listeriolysin protein) which is prototrophic for these aromatic components did not grow in the cytoplasm of the infected J774 cells, although at least 8% (8 of 91) of these bacteria were free in the cytoplasm as found by electron microscopy (Fig. 3C). The control strain *E. coli* K-12 without hybrid listeriolysin protein did not escape from the phagosome (data not shown). There remains the possibility that the expression of the hybrid listeriolysin protein and/or the translocator proteins HlyB and HlyD may inhibit the intracellular replication of *E. coli* and *S. dublin* in the J774 macrophage cells, which is not the case during in vitro growth. Alternatively, *S. dublin* and *E. coli* K-12 carrying the hybrid listeriolysin gene may be unable, for unknown physiological reasons, to grow in the host cells.

Our results clearly show that the intracellular localization of *S. dublin* can be changed from the vacuole to the cytoplasm by providing these bacteria with a cytolytic toxin. The high secretion rate of this hybrid protein observed in the *S. dublin* strain demonstrates that the *E. coli* hemolysin secretion apparatus recognizes and transports extracellular proteins from gram-positive bacteria with the same efficiency as proteins from gram-negative bacteria which are normally transported by the *sec* pathway (15), provided the N-terminal transport signal of these proteins is replaced by the C-terminal HlyA secretion signal (6). The data further show that the addition of the C-terminal HlyA signal does not interfere with the pore-forming activity of listeriolysin. This is one of the rare examples of a secreted HlyA fusion protein which is shown to be functionally active.

Isogenic listeriolysin-positive and -negative *Salmonella* strains offer the opportunity to study the function of the same set of virulence genes expressed in the vacuole or in the cytoplasm. This may also provide an interesting new approach for

TABLE 1. Hemolytic activities of supernatants and whole-cell suspensions of *S. dublin aroA* strains and *L. monocytogenes* EGD

Strain	Hemolytic activity (CHU)[a]	
	Supernatant	Whole-cell suspension[b]
L. monocytogenes EGD	8	16
S. dublin/pILH1	4	8
S. dublin/pMOhly1	ND[c]	ND

[a] Activities are given in complete hemolysis units (CHU), which are defined as the reciprocal of the highest dilution at which complete hemolysis was detected.
[b] Extracellular and membrane-bound hemolytic activity.
[c] ND, nondetectable.

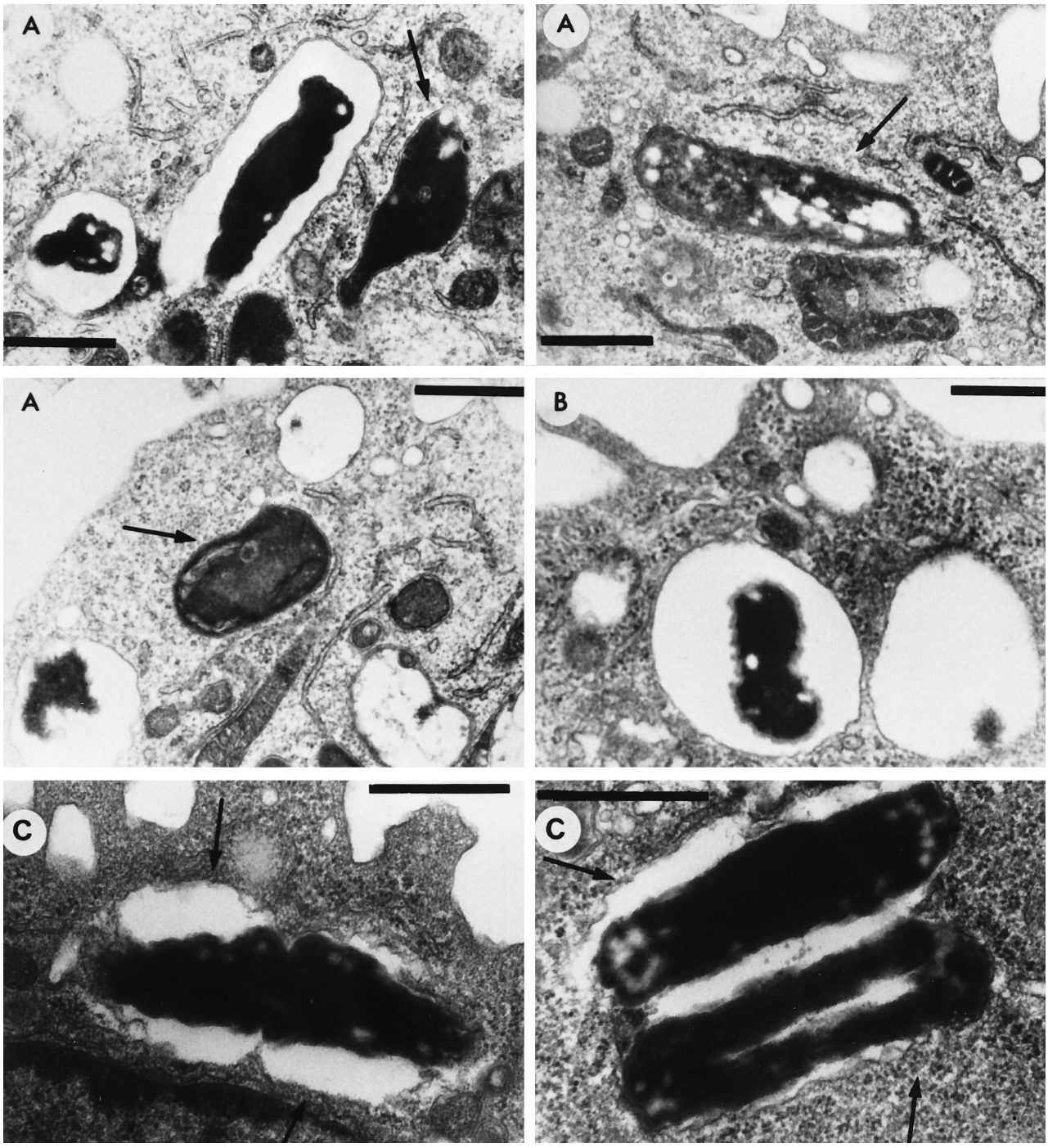

FIG. 3. Electron micrographs of *E. coli* K-12 and *S. dublin aroA* strains in J774 cells. (A) *S. dublin aroA*/pILH1 (encoding the LLO-HlyA$_s$ fusion protein); (B) *S. dublin aroA*/pMOhly1 (control carrying the vector plasmid); (C) *E. coli* K-12/pILH1. J774 cells (10^5) were infected with 10^6 bacteria. At 3 h postinfection, samples were fixed in situ, embedded, thin sectioned, and observed by electron microscopy as described previously (9). *S. dublin* or *E. coli* free in the cytoplasm are indicated by arrows. Bars = 1.1 μm.

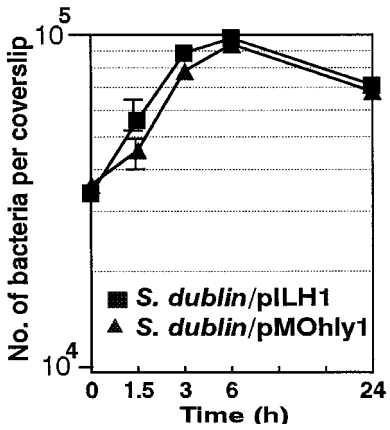

FIG. 4. Intracellular growth of *S. dublin aroA* strains in the mouse macrophage-like cell line J774. Macrophages (10^5) were infected with 10^6 bacteria. Extracellular multiplication was prevented by the addition of gentamicin (100 μg/ml) after 45 min. The number of intracellular bacteria was determined by lysing the macrophages at 1.5, 3, 6, and 24 h postinfection. (▲) *S. dublin aroA*/pMOhly1 (control carrying the vector plasmid); (■) *S. dublin aroA*/pILH1 (encoding the LLO-$HlyA_s$ fusion protein). The data represent averages for three coverslips.

analyzing antigen presentation through the major histocompatibility complex class I or class II pathway by macrophages.

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